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SHORT COMMUNICATION

Carbohydrate Concentrations in Crown Fractions from Winter Oat during Hardening at Sub-zero Temperatures

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• *Background and Aims* Contradictory results in correlation studies of plant carbohydrates with freezing tolerance may be because whole crown tissue is analysed for carbohydrates while differences exist in the survival of specific tissue within the crown. The aim of this study was to see if carbohydrate changes in tissue within oat crowns during second phase hardening (sub-zero hardening) are tissue specific.
• *Methods* The lower portion of oat (*Avena sativa*) crowns was exposed to mild grinding in a blender and the remaining crown meristem complex, consisting of tough root-like vessels, was ground in a device developed specifically for grinding cereal crown tissue. Carbohydrates were extracted by water and measured by HPLC. Carbohydrate concentrations were compared in the two regions of the crown before and after hardening at sub-zero temperatures.
• *Key Results* Fructan of all size classes except DP>6 decreased during sub-zero hardening in both stems (base of leaf sheath) and crown meristem complex. Total simple sugar increase, including sucrose, was significantly higher in the crown meristem complex than in the stem.
• *Conclusions* Results support the hypothesis that carbohydrate change in mildly frozen plants is tissue specific within crowns and underscore the need to evaluate specific tissue within the crown when correlating the biochemistry of plants with freezing tolerance.

Key words: Freezing tolerance, crown tissue, oat, *Avena sativa*, carbohydrates, fructan, apical meristem.

INTRODUCTION

The survival of cereal crops during winter depends on the ability of the below-ground portion of the stem, called the crown, to survive freezing temperatures. For this reason, the freezing tolerance of whole plants is routinely evaluated by freezing only crown tissue (Olien, 1964; Marshall, 1965).

The complexity of the crown, in terms of tissue type and vessel arrangement, has prompted several investigations into the freezing survival of specific tissues within the crown. Tanino and McKersie (1985) found that the apical meristem within wheat (*Triticum aestivum*) crowns could survive colder temperatures than the lower portion of the crown. Pearce *et al*. (1998), in a study to localize expression of cold-inducible genes within barley (*Hordeum vulgare*) crowns, demonstrated differential survival of specific tissues. Olien (1981) used histological observations of barley crowns during recovery from freezing to confirm similar differences in response within the crown. Shibata and Shimada (1986) reported that the apical meristem of *Dactylis glomerata* was less freezing tolerant than that of the lower crown. Livingston *et al*. (2005) found that the apical meristem of cold-hardened oats (*Avena sativa*) was more freezing tolerant than the crown core and that apparent barriers to freeze damage were present within the crown. These studies all indicate that the crown is a complex organ composed of tissues that differ significantly in freezing tolerance even within the same crown.

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In an attempt to explain differential tissue survival in barley crowns, Pearce *et al*. (1998) showed that a cold-induced mRNA was expressed primarily in the vascular transition zone of the crown. One of two other mRNAs was found primarily within epidermal tissue of the crown and leaves and a third mRNA was expressed only in the inner layers of the cortex and cell layers found in proximity to the vascular transition zone (Pearce *et al*., 1998). Houde *et al*. (1995) used immunocytochemistry to localize a late embryogenesis abundant protein (LEA) family (WCS120) to the vascular transition zone of wheat crowns but none was found in mersitems or in mature xylem. While not directly related to cold-hardening, Sossountzov *et al*. (1991) demonstrated that expression of a lipid transfer protein (LTP) during seed maturation was highest in the outer epidermis of coleoptiles and in leaf veins of maize. They determined that *LTP* gene expression was not just tissue specific but was also cell specific and time specific. These studies illustrate the importance of considering specific tissue and possibly even specific cells when attempting to determine cause and effect of various metabolic changes in plants during cold hardening and subsequent freezing.

Cold-hardened plants that are subjected to a mild freeze (–3 °C, 3 d) have long been known to acquire freezing tolerance beyond that achieved by hardening at above freezing temperatures. This hardening at below freezing temperatures has been called ‘second phase hardening’ (here it is called ‘sub-zero hardening’) and has been demonstrated in wheat (Trunova, 1965), rye (*Secale cereale*) (Olien, 1984),

barley and oat (*Avena sativa*) (Livingston, 1996), alfalfa (Castonguay, 1993) and *Arabidopsi*s (D. Livingston, unpubl. res.).

Rapid carbohydrate shifts (within 24 h) have been demonstrated in winter cereal crowns exposed to sub-zero hardening (Olien and Lester, 1985; Livingston, 1996). Livingston and Henson (1998) reported that sugar, as well as fructan, increased significantly in apoplastic fluid from a winter-hardy oat during such a freeze. In addition, they reported a significant increase in the activity of fructan exohydrolase and invertase in the apoplast. Subsequent experiments, however, suggested that apoplastic fluid was probably extracted from stem tissue, and concentric leaf-bases directly above the meristematic regions and not from the base of the crown where survival is crucial (Olien, 1981; Tannino and McKersie, 1985; Livingston *et al*., 2005). Because apoplastic carbohydrate changes during sub-zero hardening were not correlated with freezing tolerance in oat cultivars (Livingston and Premakumar, 2002) the possibility was considered that carbohydrate shifts during sub-zero hardening were tissue specific. The purpose of this research was to see if differences in carbohydrates could be detected in subfractions of crown tissue from a winter-hardy oat before and after it had been hardened at sub-zero temperatures.

MATERIALS AND METHODS

Plant culture

Seeds of oat (*Avena sativa* 'Wintok') were grown in Scotts Metromix 220 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) in plastic tubes (2·5 cm diameter × 16 cm height) with holes in the bottom to allow drainage. The tubes were suspended in a grid that held 100 tubes. Plants were watered three times weekly with a complete nutrient solution (Livingston, 1991) and flushed three times weekly with tap water. Plants were grown for 5 weeks at 13 °C day and 10 °C night temperatures with a 12-h photoperiod at 280 μmol m^{-2} s^{-1} PAR (80 % cool fluorescent and 20 % incandescent). After 5 weeks, plants were transferred to a chamber at 3 °C with a 12-h photoperiod at 300 μmol m^{-2} s^{-1}. Plants remained under these conditions for 3 weeks; this constituted cold hardening.

Plants were washed free of soil, trimmed to 5-cm shoots and 1-cm roots and placed in plastic bags in a freezer at −3 °C for 3 d in the dark. This constituted the sub-zero hardening treatment.

Histology

The crown, measuring about 2–3 cm above the root–shoot junction (Fig. 1D) was placed in FAA containing 18 : 1 : 1 parts of 70 % ethyl alcohol : glacial acetic acid : formalin. Samples were kept at room temperature for 48 h and transferred to 70 % alcohol and kept at 4 °C until they were processed for dehydration and embedding.

Dehydration, infiltration, embedding and staining

The dehydration of samples was done according to the procedures outlined in Johansen (1940) using graduated solutions of EtOH and TBA. Fully infiltrated tissues were embedded in Parapast Plus and kept refrigerated until sectioned. Embedded sample blocks were sectioned in a Reichert-Jung 2050 rotary microtome at a thickness of 15 μm. The resulting paraffin ribbon containing serial sections was placed on a glass slide coated with Haupt's adhesive (Johansen, 1940), flooded with 3 % formalin, and transferred to a slide warmer at 41 °C. Dried slides were stored at room temperature until stained. The slides were left overnight in dishes containing xylene to remove paraffin before sections were stained. A triple stain with Safranin, Fast green and Orange G was used as described by Johansen (1940). A coverslip was added to the slides with one or two drops of Permount.

Observation of sections

After drying for 1 or 2 d, mounted sections were viewed on a Zeiss photomicroscope III to observe differences among the samples. Representative sections were viewed under a Wild Heerbrug wide-angle dissecting microscope with bottom lighting. Photographs were taken with a Sony DSC707 digital camera attached to the microscope.

Crown partitioning

Plants were taken for partitioning before and after sub-zero hardening. They were washed free of soil and roots and were trimmed as close to the base of the stem as possible. One centimetre of the base of the crown was cut (Fig. 1E), and placed on ice until ten plants were processed. When all ten plants had been cut the crown-base tissue was ground in a cold blender in 20 mL ice water for 30 s. The crown meristem (CM) complex (Fig. 1F) from each plant was removed by tweezers, blotted dry, weighed and then ground in a grinder developed specifically for carbohydrate analysis of crown tissue from cereal crops (Livingston, 1990). The leaf-base tissue remaining in the blender was transferred to a beaker, heated to 95 °C for 20 min and prepared for HPLC analysis. The ground CM complex was transferred to a beaker and treated identically to the leaf-base tissue. This procedure was repeated three times. All data are a mean of three replications and each replicate consisted of ten plants.

Carbohydrate analysis

Ground and heated CM complex and leaf-base tissues were filtered through 0·45-m filters and injected into a BioRad 42A HPLC column. Fructans were separated according to size class using a modified Bio-Rad (Richmond, CA, USA) Aminex HPX-42A (silver based) analytical HPLC column (7·8 × 300 mm). This column was permanently modified by passing 0·5 M $NaNO_3$, at a rate of 2 mL min^{-1}, through the column for approx. 18 h. This treatment eliminated on-column hydrolysis of smaller fructans and sucrose and improved the resolution of smaller sugars. Resolution of larger (DP5 and DP6) fructans, however, was slightly reduced in the modified column. As the samples were not desalted prior to injection, a cation and anion exchange guard-column was used immediately preceding the analytical column to prevent co-elution of

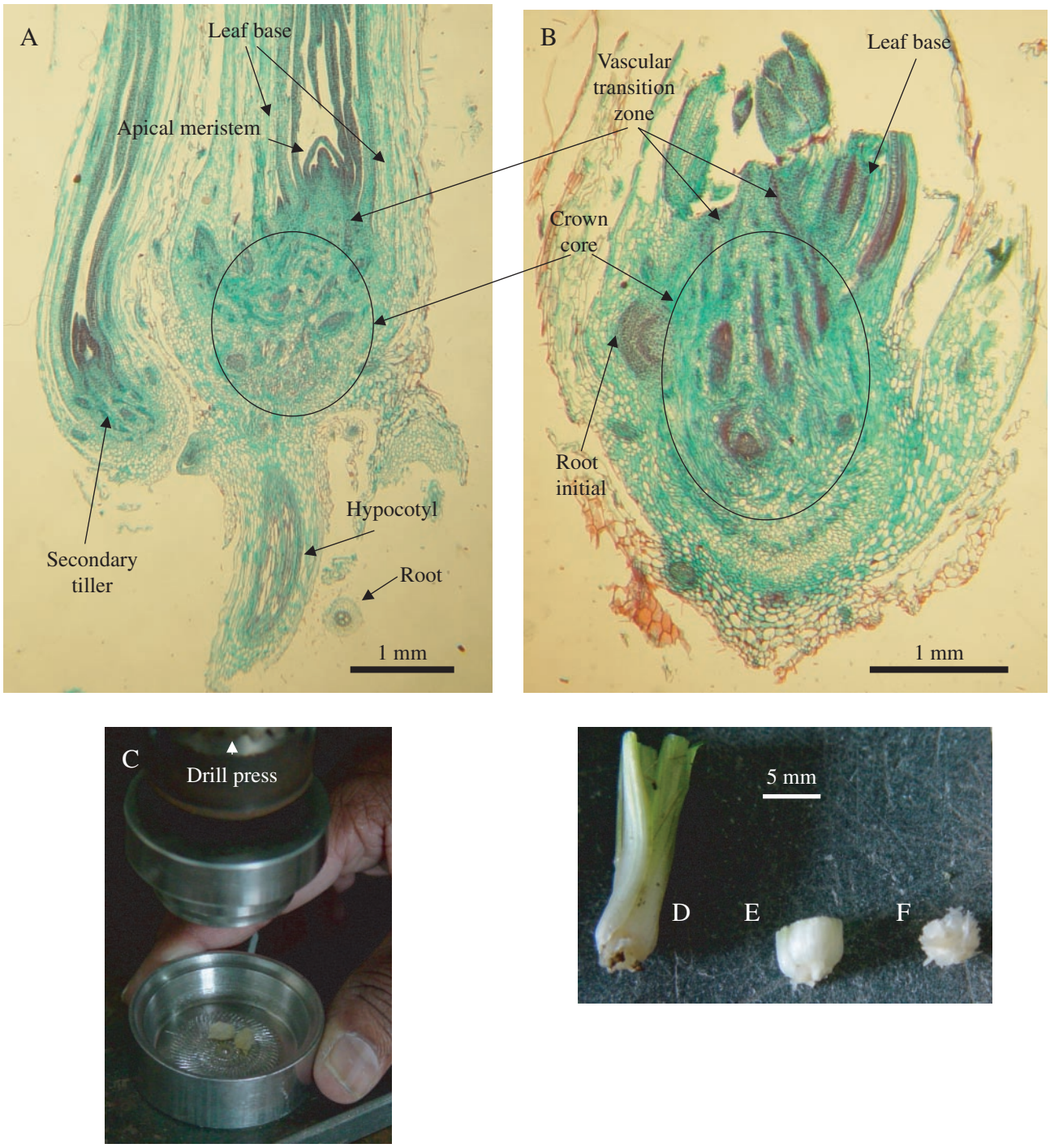

FIG. 1. (A) Longitudinal section of a paraffin-embedded crown of oat that was cold hardened at 3 °C for 3 weeks. (B) Crown meristem (CM) complex after grinding in a blender. The sections in A and B were subjected to a triple stain of Safranin, Fast green and Orange G. (C) The CM complex ready for grinding in a device mounted in a drill press that was developed specifically for grinding cereal crowns (Livingston, 1990). (D) Intact crown; (E) base of crown cut in preparation for initial grinding in blender; (F) the CM complex after grinding in blender and before complete grinding in device shown in C.

small ionic compounds with carbohydrates. The mobile phase was HPLC-grade water at a flow rate of 0·4 mL min^{-1}.

Statistical analysis

The experiment was conducted as a randomized complete block design with three replicates and with hardening treatment as the main plot and tissue as sub plot. The analysis of variance (Table 1) was calculated using MSTAT (Michigan State University, East Lansing, MI). Carbohydrate experiments were repeated several times with minor differences between experiments but none that contradicted the hypothesis under consideration.

TABLE 1. *Carbohydrate concentrations (mg g^{-1} f. wt) in crown meristem (CM) complex and leaf-base ('leaf b') of oat crowns before and after sub-zero hardening*

		Fructan										
Hardening	Tissue	DP>6	DP6	DP5	DP4	DP3	Suc	Glu	Fru	fn	SS	Total
C	CM	17	5·5	5·8	6·3	4·2	12	2·8	1·8	39	17	56
SZ	CM	16	4·5	4·1	3·5	1·5	20	3·5	3·2	29	27	56
Difference		−1	−1·0	−1·7	−2·8	−2·7	8	0·7	1·4	−10	10	0
C	Leaf b	13	5·1	6·0	6·9	4·8	11	4·5	1·1	36	17	52
SZ	Leaf b	12	3·8	3·6	3·1	1·2	8·1	4·0	3·0	24	15	39
Difference		−1	−1·3	−2·4	−3·8	−3·6	−2·8	−0·5	1·9	−12	−2	−13
Statistics												
Hard		n.s.	n.s.	*	*	*	n.s.	n.s.	**	0·07	n.s.	n.s.
Tissue		*	n.s.	n.s.	n.s.	n.s.	**	*	*	n.s.	**	*
H × tissue		n.s.	n.s.	n.s.	n.s.	n.s.	**	0·08	n.s.	n.s.	**	0·06

Each data point is the mean of three replicates.

n.s., Non-significant at $P = 0{\cdot}05$; *, **, significant at $P = 0{\cdot}05$, 0·01, respectively.

H × tissue, the interaction of tissue type with change during sub-zero hardening; Hard, hardening conditions; C, cold hardened (3 weeks at 3 °C); SZ, sub-zero hardening (3 d at −3 °C); DP, degree of polymerization; fn, total fructan; SS, simple sugar (glucose+fructose+sucrose).

RESULTS AND DISCUSSION

The crown tissues under consideration are shown in Fig. 1. The upper portion analysed (not shown because it was removed by grinding in a blender) was a part of the stem consisting of concentric leaf-bases with their respective sheaths (compare with intact crown in Fig. 1A) and the lower portion is referred to as the 'crown meristem (CM) complex' (Fig. 1B and F). This complex contains root and apical meristems as well as support tissue consisting of intertwining vessels resembling those in the root, but interspersed with parenchyma cells. This tissue, which was illustrated in detail elsewhere (Livingston *et al*., 2005), is being called the 'crown core'. The procedure for partitioning the crown occasionally removed the tip of the apical meristem (compare Fig. 1A with Fig. 1B) and did not always perfectly remove leaf-base tissue (Fig. 1B).

The success of this method of partitioning the crown is based on the difficulty of grinding (in a commercial blender) the CM complex as compared with the leaf-base. This allowed most of the leaf-base tissue to be ground away, leaving the unground portion of the crown (CM complex; Fig. 1B and F) clearly visible for removal by hand and subsequent grinding separately. The tough nature of the CM complex is confirmed by the necessity of developing a grinder specifically for this tissue (Fig. 1C; Livingston, 1990).

Carbohydrates

Total carbohydrate was significantly lower in leaf-bases than in the CM complex (Table 1). Smith (1967) reported lower percentages of total carbohydrates in stem bases (this presumably corresponds to our 'CM complex') as compared with leaf sheaths in bromegrass and timothy. Volenec and Nelson (1984) reported that sugars and fructans were highest in meristematic tissue of tall fescue and that both progressively decreased as they sampled further up the stem. Heterogeneity between cell types in barley was reported (Koroleva *et al*., 2002) for sugars as an explanation for differences in transcription control and enzyme activity. Very low concentrations of glucose, fructose, sucrose and fructan were found in epidermal cells of barley leaves with much higher concentrations in mesophyll and bundle sheath that had been cooled (Koroleva *et al*., 1998).

The biggest difference between the leaf-base and CM complex tissue occurred after sub-zero hardening and the biggest contributor to total carbohydrate change was fructan (Table 1). Fructan exohydrolase activity increased in apoplastic fluid of crown tissue during sub-zero hardening (Livingston and Henson, 1998) and was considered the primary cause of fructan decrease and concomitant increase of simple sugars in apoplastic fluid. It was not possible to centrifuge apoplastic fluid from the CM complex at speeds that did not damage cells (data not shown), presumably because of the tightly packed nature of cells in this part of the crown, especially in the crown core. It is therefore probable that much of the increase in sugars during sub-zero hardening (Table 1) was symplastic. Carbohydrates have long been implicated in protection from freezing injury and numerous protective mechanisms that could be operative apoplastically or symplastically have been proposed for various sugars (for several early reports, see Steponkus, 1968; Alden and Herman, 1971; Santerius, 1973; Olien and Lester, 1985). Recent studies have demonstrated a stabilizing effect on various membranes by trehalose (Crowe *et al*., 2001; Crowe, 2002), raffinose (Hincha *et al*., 2003), sucrose (Ottenhof *et al*., 2003; Hincha and Hagemann, 2004) as well as fructo- and gluco-oligosaccharides (Hincha *et al*., 2002). In addition to direct membrane stabilization, sucrose has been implicated as a regulatory compound for cold-induced expression of non-specific LTP and dehydrin in barley cell cultures (Tabaei-Aghdaei *et al*., 2003).

Hydrolase activities were not measured in the two tissues here, so it is not known whether the decrease in fructan (Table 1) during sub-zero hardening was due to hydrolysis or possibly to translocation induced by freezing. The

increase in simple sugars during sub-zero hardening (Table 1) was highly significant in CM complex tissue but was unchanged in leaf-bases, suggesting fructan hydrolysis in the CM complex, especially since the increase in simple sugars (glucose + fructose + sucrose) was nearly identical to the decrease in fructan. In addition to sugars, total protein content was nearly two-fold higher in the CM complex from sub-zero-hardened plants (D. Livingston, unpubl. res.) with a significantly greater response to sub-zero hardening in CM tissue than in the leaf base. Proteomic analysis is being conducted on crown tissue to determine specific proteins that are up- and down-regulated during sub-zero hardening.

Leaves and roots are the most tender tissues exposed to cold during winter and frequently are killed (Olien, 1981). Provided the meristematic regions of the crown (CM complex) survive injury, a complete plant will regenerate in the spring (Marshall, 1965; Olien, 1981). It is possible that sugars in the CM complex remain symplastic and provide protection to this crucial part of the crown in the form of membrane stabilization. It is likely that after sub-zero hardening some sugars are apoplastic in the CM complex but, until techniques are developed to sample apoplastic fluid from the CM complex, this cannot be confirmed. Further fractionation of the CM complex is certainly possible and is being investigated.

CONCLUSIONS

Results reported here demonstrate tissue specificity for carbohydrates before and after a mild freeze. This indicates that biochemical acclimation during sub-zero hardening occurs at different rates in specific tissue than in an average of all the tissue in crowns. Differences in the biochemistry of the two regions of the crown suggest that analysis of specific tissue in the crown is crucial to a complete understanding of mechanisms of whole-plant freezing survival.

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